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THE OCCURRENCE OF A SOLUBLE LIPASE IN BROTH CULTURES OF TUBERCLE BACILLI AND OTHER ACID-FAST BACTERIA

STUDIES IN ACID-FAST BACTERIA. VI *

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The growth of two rapidly growing strains of human tubercle bacilli in media of varied composition showed consistently two distinct phases in their metabolism: an initial phase characterized by rapid vegetative activity and progressive proteolysis, as indicated by a steady increase in ammonia formation; and a second phase marked by a progressive decrease in vegetative activity, and by recessive changes in metabolism, during which the ammonia accumulated during the initial phase gradually disappeared from the culture media (These Studies, I-V).

This second phase, which appears to begin rather regularly after three weeks' growth in ordinary media with the organisms studied, is of unknown causation. It is quite probable that the accumulation of waste products plays a prominent part in restricting the activity of the organisms, but the simple restriction of bacterial growth *per se* does not explain the associated phenomenon of the recession of the ammonia which has been produced during the period of rapid development. Furthermore, attempts to correlate the recession of ammonia with the presence of certain substances in the media, in which these observations were made, were unsuccessful, for even in a culture solution as simple in composition as $(NH_4)_2HPO_4$, NaCl, and dextrose, the same phenomenon was observed, suggesting strongly that the explanation of the recession is to be sought for in the organisms themselves. This at once focuses attention on the possibility of ferments, exo- or endo-cellular, being concerned, the entire process, perhaps, being one in which autolysis plays a prominent part.

The tubercle bacillus and other acid-fast bacteria are notoriously resistant to solvents which will promptly destroy other organisms not acid-fast. In the animal body, similarly, these bacteria are resistant to

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lytic powers which usually suffice to destroy corresponding numbers of the ordinary organisms. Fats and waxes, which are present in considerable amount in the substance of these acid-fast bacteria, appear to confer this resistance to solution on these organisms. Consequently, the removal or modification in composition of these substances would appear to be an essential factor in the autolysis of the acid-fast type of bacteria.

Comparatively little appears to have been written on the subject of fat-splitting by bacterial ferments. It is claimed by Rubner[1] and others that the microbic decomposition of fats with the liberation of fatty acids and glycerin is in reality a "fat fermentation" brought about by the direct activity of the protoplasm of the organism, not to a definite fat-splitting ferment elaborated by them. On the other hand, Sommaruga[2] and others appear to have demonstrated lipases in several types of bacteria; Carriere[3] demonstrated a lipase in the bodies of six-months-old tubercle bacilli, but not in the media in which they were grown; Wells and Corper[4] have also demonstrated lipases in cultures of tubercle bacilli.

In order to determine whether esterases or lipases play a part in the development of the tubercle bacillus in artificial media, a series of cultures of various strains of acid-fast bacilli of various ages, including not only human tubercle bacilli of various degrees of virulence, but also bovine and avian tubercle bacilli as well, and, in addition, certain other acid-fast bacteria, including the grass bacillus, the leprosy bacillus isolated by Duval, and the smegma bacillus, were examined for evidence of ester- and fat-splitting ferments. The organisms investigated, without exception, grew as a firm, tenacious pellicle on the surface of the medium, leaving the underlying fluid perfectly clear. This clear fluid, free from bacteria, was used in these experiments.

The technic adopted was as follows: One cubic centimeter of this clear fluid underlying the bacteria was placed in a large, clean test-tube with 10 c.c. of freshly distilled water, using sterile precautions; 0.25 c.c. of ethyl butyrate (neutral in reaction) and 0.5 c.c. of toluene were then added, and the whole shaken one hundred times in order to produce a well-mixed emulsion.[5] Two drops of phenolphthalein were then added and the mixture brought to the neutral point by titration with N/50 acid or alkali, depending on the reaction. This mixture was then stoppered and incubated at 37 C. for twenty-four hours, and again brought to neutrality with N/50 alkali. The increase in acid-

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1. Arch. f. Hyg., 1900, 38, p. 67.
2. Ztschr. f. Hyg. u. Infektionskrankh., 1890, 18, p. 441.
3. Compt. rend. Soc. biol., 1901, 53, p. 320.
4. Jour. Infect. Dis., 1912, 11, p. 388.
5. Jobling and Bull. Jour. Exp. Med., 1912, 16, p. 483.

ity, measured in terms of N/50 NaOH, was taken as a measure of the lipolytic activity of the culture.

All of the cultures tested for lipase in this series were grown in broth, uniform in composition, in volume (100 c.c.), and with the same area of free surface. It will be seen that the increase in acidity observed represents, theoretically, 1/100 of the total lipolytic activity of the particular culture under discussion, or, better, 1/100 of the amount of acid which would be liberated by the entire culture in twenty-four hours under the given conditions, for it will be remembered that but 1 c.c. of the culture was used for the test: the results, therefore, are comparative, and roughly quantitative.

Almost without exception, the culture media in which these acid-fast bacteria had grown were rather dark-colored, and this color was sufficient in undiluted solutions to obscure somewhat the end point to phenolphthalein during titration. For this reason 1 c.c. of the culture medium was diluted with 10 c.c. of freshly boiled, distilled water. This dilution accomplished three results: boiling the water drove out all CO_2, and the re-entrance of CO_2 was prevented by the layer of toluene which formed a seal on the surface; diluting the broth with 10 c.c. of water also diluted the color proportionately, so that the end point was very much more distinct; finally, the dilution also prevented any considerable concentration of products of ferment activity which might otherwise tend to arrest the process.

It was found by experiment that 0.25 c.c. of ethyl butyrate was sufficient to react with all the lipase present in the media. This ethyl butyrate was freed from acid before it was added to the media.

Toluene was added for two reasons: first, to prevent the absorption of CO_2 from the air and thus increase the acidity of the medium; and, secondly, to restrain the development of any extraneous bacteria which might have inadvertently reached the solution during the process of preparation and titration: 0.5 c.c. of toluene was sufficient to accomplish these two results.

When the diluted broth, ethyl butyrate, and toluene were prepared in the manner indicated above, the tube in which they were placed was agitated thoroughly in order to mix the substances as intimately as possible.

As a rule, the initial reaction of the lipase solution thus prepared was approximately normal to phenolphthalein at the start, but occasionally cultures were found in which there was a slight initial acid or alkaline reaction. In every instance, the reaction was adjusted to a faint pink by the addition of N/50 acid or alkali, as the case might be, prior to incubation.

At the end of twenty-four hours' incubation at 37 C. the tubes were again shaken and the reaction determined by titration with N/50 NaOH. Incubation was continued for seventy-two hours in the early determinations to measure the total ester-splitting power of the media, but the increase in acidity occasionally detectable after twenty-four hours was found to be so slight it was disregarded in these tables, which are designed to show the presence, rather than the exact measure, of the lipase produced by these organisms. Indeed, duplicate determinations on the same sample indicate that the inherent errors of the method (indicator error and error of sampling) practically compensated for these very slight increases which were occasionally detected after seventy-two hours' incubation. The average difference between duplicate determinations is about 0.1 c.c. N/50 NaOH, and this figure appears to represent the average error of the method. The increase in acidity in cubic centimeters of this solution was taken as a measure of the activity of the lipase in the culture medium. It should again be remarked parenthetically that the theoretical lipase activity of the entire culture would be one hundred times the amount determined in the process described above, for but 1/100 of the medium was used for this determination.

Throughout these experiments, controls were made in the following manner. A media control in which 1 c.c. of uninoculated (sterile) media of the same composition as that of the cultures was prepared in precisely the manner outlined above, the only difference being that the media control was free from any products of bacterial development. A second media control was made in which the broth containing the lipase was incubated under parallel conditions without the ester. This latter control was designed to show any changes in reaction which might take place spontaneously in the medium in the absence of the ester. A third ester control was used in which the ester was prepared with toluene and distilled water, but to which no broth, either inoculated or uninoculated, was added. These controls were incubated under precisely the same conditions as the lipase solutions, and, without exception, these controls have invariably been neutral at the end of twenty-four hours: that is to say, there was absolutely no increase in acidity in these controls which contained uninoculated broth and an ester, inoculated broth and no ester, and an ester alone. Consequently, the accumulation of acid observed in these experiments after twenty-four hours is attributable to the splitting of the ethyl butyrate by a reactive substance developed in these media during the growth of the organisms, resulting in the liberation of butyric acid. From the conditions of the experiment it would appear that this substance, which split the butyric ether, was a fat-splitting ferment. It might be objected that a ferment which splits ethyl butyrate should be termed an esterase and not a lipase: such esterases, which do not decompose complex glycerids, occur in the human body. Achard and Clerc, and Saxl[6] have shown that an esterase which splits monobutyrin is present in the blood serum of man; they were, however, unable to demonstrate the presence of a true lipase. In order to answer this objection, the same series of experiments was carried out, using castor oil in place of ethyl butyrate as a substrate. The castor oil was first neutralized and emulsified by the cautious addition of N/10 NaOH until a faint permanent pink reaction resulted, according to the method of Kanitz.[7] This emulsion remained permanent for several days: 0.5 c.c. of this emulsion was added to a series of tubes prepared precisely as those described above, with the same controls.

Culture "597" is a rapidly growing human tubercle bacillus which was obtained from the American Museum of Natural History of New York through the kindness of Professor Winslow. Culture "W" is an avirulent, rapidly growing, human tubercle bacillus which came from the laboratory of Professor Wherry of the University of Cincinnati. Culture "257" is also a human tubercle bacillus intermediate in its growth and virulence between the rapidly growing strains just described and cultures human and human "X," which were obtained from Prof. Theobald Smith of the Harvard University Medical School. These latter cultures, human and human "X," are experimentally pathogenic for guinea-pigs. The bovine tubercle bacillus came originally from the Bureau of Animal Industry, as did the avian culture. For these last two organisms we are indebted to Dr. Enos Day, Director of the Pathological Laboratory at the Chicago Stock Yards. The leprosy bacillus was one isolated by Duval. The smegna bacillus and Grass bacillus III were cultures the histories of which are unknown to us.

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[6] Achard et Clerc, Compt. rend Soc. de biol., 1902, 54, p. 1144. Saxl, Biochem. Ztschr., 1908, 12, p. 343.

[7] Ztschr. f. physiol. Chem., 1905, 46, p. 482.

Table 1 shows the organism, the medium in which the organism was grown, the age of the culture, and the amount of acid in terms of N/50 NaOH, which developed when 1 c.c. of each of these cultures, respectively, was incubated twenty-four hours under the conditions outlined above. It will be observed that, as a general rule, the degree of acidity was somewhat greater when ethyl butyrate was used as a substrate than when castor oil was used for the same purpose, under the same conditions. While the amounts of acid produced by the bacteria shown in this table are not large, it must be remembered that the theoretical acidity, which would be developed by the entire culture under parallel conditions, would have been 100 times the values given. It would appear, therefore, that, without exception, these acid-fast organisms produce, as the result of their growth in artificial media, a substance which is in solution and which possesses the property of splitting both ethyl butyrate and castor oil. From the nature of this splitting it would appear justifiable to designate this substance a lipase.

TABLE 1
AMOUNT OF ACID PRODUCED

Organism	Medium	Age of Culture	C.C. N/50 NaOH	
			Ethyl Butyrate	Castor Oil
B. tuberculosis-Human 597	Mannite broth	3 months	2.10	1.85
B. tuberculosis-Human W	Glycerin broth	4 months	1.25	1.15
B. tuberculosis-Human X	Glycerin broth	4 months	1.35	1.20
B. tuberculosis-Human	Glycerin broth	4 months	1.80	1.65
B. tuberculosis-Human 257	Glycerin broth	1 month	1.30	1.35
B. tuberculosis-Avian	Glycerin broth	1 month	1.25	1.35
B. tuberculosis-Bovine	Glycerin broth	2 months	1.00	0.95
B. leprae (Duval)	Glycerin broth	1 month	0.35	0.20
B. smegmatis	Glycerin broth	1 month	1.00	0.90
B. grass IV	Glycerin broth	1 month	1.20	1.15
Control (media)	Glycerin broth	—	0.00	0.00
Control (ester)	—	—	0.00	0.00

In order to throw some light on the physical properties of this lipase, a series of experiments was made with the same organisms, using different cultures, however, to determine the degree of resistance of this lipase to heat. A series of initial experiments was made in which it was found that heating to 60 C. for thirty minutes had little or no effect on the activity of the lipase. A second series of experiments was made in which 1 c.c. of the respective culture fluids, suspended in 10 c.c. of distilled water, was heated at the temperature of boiling water for fifteen minutes, and then, after the addition of the ester and toluene, compared under precisely the same conditions with unheated cultures of the same bacteria. It will be seen from Table 2 that even heating to 100 C. for fifteen minutes is without noteworthy effect on the activity of this lipase. At first sight, this unexpected resistance of the reactive substance to heat would seem definitely to eliminate a ferment as the cause of this splitting of the ester and castor oil, for the vast majority of ester- and fat-splitting ferments which have been studied up to the present time have been found to be thermolabile, losing their activity at temperatures above 60 C. Carrière found that the lipase of the tubercle bacilli, which he studied, was thermolabile. Loevenhart,[8] however, has shown that the lipase of the pancreas, when dried, can be heated at 110 C. for one and one-half hours with the loss of about 50 percent of its activity when measured on ethyl butyrate, altho its action on olive oil remained undiminished. Abbott and Gildersleeve[9] have shown that certain proteolytic enzymes of bacterial origin may be heated in the moist state to a temperature of 100 C. for fifteen to thirty minutes without impairing their characteristic function. Other observers also have found that ferments of bacterial origin may be thermostabile. With these observations in mind it appears logical to attribute the splitting of ethyl butyrate and castor oil observed above to the action of a true lipase.

TABLE 2
EFFECT OF HEAT ON THE LIPASE

Organism	Medium	Age of Culture, Days	C.C. N/50 NaOH Ethyl Butyrate	
			Unheated	Heated
B. tuberculosis W	Plain broth	21	1.40	1.35
B. tuberculosis W	Dextrose broth	21	1.40	1.30
B. tuberculosis W	Mannite broth	21	1.45	1.25
B. tuberculosis W	Glycerin broth	21	1.35	1.30
B. leprae (Duval)	Plain broth	21	—0.15	—0.25
B. leprae (Duval)	Dextrose broth	21	—0.20	—0.20
B. leprae (Duval)	Mannite broth	21	—0.15	—0.20
B. leprae (Duval)	Glycerin broth	21	—0.10	—0.25
B. smegmatis	Plain broth	21	1.20	1.20
B. smegmatis	Dextrose broth	21	1.20	0.80
B. smegmatis	Mannite broth	21	1.40	1.15
B. smegmatis	Glycerin broth	21	1.50	1.20
B. grass IV	Plain broth	21	1.30	1.10
B. grass IV	Dextrose broth	21	1.25	—
B. grass IV	Mannite broth	21	1.10	1.40
B. grass IV	Glycerin broth	21	1.20	1.30

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[8] Jour. Biol. Chem., 1907, 2, p. 451.
[9] Jour. Med. Research, 1903, 10, p. 61.

Most ferments are said to be non-diffusible. In order to test the diffusibility of the lipase found in the cultures of acid-fast bacilli, two distinct methods were tried: diffusion through agar and diffusion through collodion membranes.

To determine the diffusibility of the lipase through agar, a series of test-tubes of uniform diameter (1.5 cm.) and length (15 cm.) were bent at right angles midway of the long axis, so that the resulting formation resembled a capital "L." Each test-tube was then filled to about two-thirds of its length with glycerin agar, slanting the agar in that portion of the tube nearer the open end, and then inoculating the slanted surface with one of the rapidly growing human tubercle bacilli. In this way a maximum surface growth of the organism was obtained. These cultures were incubated and examined at regular intervals as follows: One of the bent agar tubes was broken in such a manner that the agar in the closed end was removed absolutely free from the growth on the slanted surface, thus eliminating bacteria. This agar was divided into two parts, A and B; A being nearer the slanted surface, B being more remote from the slanted surface. These portions of agar were then macerated with sterile precautions in 5 c.c. of sterile distilled water., 0.25 c.c. of ethyl butyrate added together with 0.5 c.c. of toluene, shaken, neutralized, and the whole then incubated at 37 C. for twenty-four hours. If any lipase had diffused from the bodies of bacteria on the slanted surface of the agar through to the closed arm from which these samples were taken, the customary splitting of the ethyl butyrate and castor oil should take place. A series of twelve such determinations was made, growths from one week to three months being examined in the series, with absolutely uniform negative results; that is to say, this experiment would indicate that an active lipase had not diffused in demonstrable amounts into and through the agar. The organisms grown on the surface, however, contained lipase in every instance. This experiment was not deemed conclusive, for it is conceivable that the composition and reaction of the agar may have inhibited the action of the ferment.

Another attempt was made to determine the diffusibility of the lipase by suspending a collodion sac, 1 cm. in diameter and 10 cm. long, filled with broth known to contain an active lipase, in a test-tube 2 cm. in diameter, the space between the collodion sac and the walls of the tube being occupied by sterile distilled water, with a layer of toluene overlying both the contents of the sac and the water outside the sac.

Even after a week's incubation at 37 C., the fluid outside the collodion membrane being tested daily after addition of ethyl butyrate in the usual manner, no evidence of lipase activity could be detected outside the collodion sac. The fluid within the collodion membrane showed uniformly strong lipolytic action both on ethyl butyrate and castor oil. This experiment was repeated in detail, using the bodies of tubercle bacilli, which had been washed thoroughly and then killed with toluene water, in place of the broth culture containing the lipase; the supposition being that, as the tubercle baccilli autolyzed, a certain amount of lipase might escape from them and diffuse through the collodion membrane into the surrounding distilled water. Here again the results were uniformly negative, altho lipase of considerable strength could be demonstrated in the fluid inside the collodion membrane, where, it will be remembered, the tubercle bacilli were undergoing autolysis.

CONCLUSIONS

The evidence indicates that a variety of acid-fast bacteria, including various strains of the human tubercle bacillus, the bovine, and avian tubercle bacilli, as well as the leprosy, smegma, and grass bacilli, form lipase during their growth in glycerin broth.

The lipase is present in the medium free from the bacteria.

The lipase resists an exposure of fifteen minutes to 100 C. in the moist state without appreciable diminution in its activity.

The lipase formed by these organisms appears to be non-diffusible, at least in an active state, through either agar or a collodion membrane.